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Cell attachment and fibrinogen binding properties of platelet and endothelial cell thrombospondin are not affected by structural differences in the 70 and 18 kDa protease-resistant domains

Philippe Clezardin[+], Marie-Claude Bourdillon[+], Nikolas R. Hunter and John L. McGregor[+*]

[+]*INSERM U63, Laboratoire d'Hémobiologie, Faculté de Médecine A. Carrel, rue G. Paradin, F-69372 Lyon, France, MRC/SNBTS Blood Components Assay Group, 2 Forrest Road, Edinburgh EH1 2QW, Scotland* and **Stanford Medical School, Division of Hematology, Stanford, CA 94305, USA*

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Structural differences between platelet and endothelial cell thrombospondin (TBSP) were found in two protease-resistant domains (70 and 18 kDa). The 70 kDa fragment is involved in the binding of TBSP to fibrinogen and the 18 kDa fragment in the attachment to various cultured cells. Despite these structural differences, platelet and endothelial cell TBSP bound with the same affinity to fibrinogen and mediated the attachment of smooth muscle cells but not of endothelial cells.

Thrombospondin; ELISA; Adhesion assay; (Human endothelial cell, Platelet)

1. INTRODUCTION

Thrombospondin (TBSP) is a glycoprotein secreted by a wide range of cells including platelets, endothelial and smooth muscle cells (review [1]). This glycoprotein is composed of several protease-resistant domains which bind specifically to different proteins including fibrinogen [1]. In addition TBSP is incorporated into the extracellular matrix [2] and appears to mediate attachment of platelets and a number of cultured cells (fibroblasts, squamous carcinoma, epithelial and smooth muscle cells) [3,4].

We reported recently [5] that human endothelial cell TBSP is more resistant to proteolysis than platelet TBSP. However, the molecular basis for this increased resistance to proteolysis of endothelial cell TBSP is unknown. In this study, structural differences between platelet and endothelial cell TBSP were found in two protease-resistant domains of TBSP (70 and 18 kDa), one of which (70 kDa) is involved in the binding of TBSP to a number of adhesive proteins including fibrinogen [6] and the other (18 kDa) in the adhesion to various cultured cells [7]. In view of the above structural differences which could affect the TBSP functions, we have therefore, in the second part of this study, compared the binding of fibrinogen and cultured cells to platelet and endothelial cell TBSP.

Correspondence address: P. Clezardin, INSERM U63, Laboratoire d'Hémobiologie, Faculté de Médecine A. Carrel, rue G. Paradin, F-69372 Lyon, France

2. MATERIALS AND METHODS

Endothelial cells from human umbilical cord veins and smooth muscle cells from human aorta were cultured using methods and materials previously described [5,8]. Human endothelial cell and platelet thrombospondin (TBSP) were purified as outlined in [5]. Both TBSP preparations were >95% pure when analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (not shown).

Thermolysin digestion was carried out at 37°C for 5 and 90 min and at a protease to TBSP ratio of 1:500 (w/w). After incubation, digestion of TBSP was terminated by the addition of one-quarter volume of SDS-PAGE sample buffer and heating to 100°C for 90 s [9]. Undigested and digested TBSP samples were then subjected to electrophoresis on 10% SDS-

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polyacrylamide gels. Gels were fixed in 40% methanol-7% acetic acid and silver-stained [10].

Densitometry tracings of silver-stained gels were obtained on a Vernon (PHI) densitometer as described in [11].

For the immunodetection of the TBSP complex formation with fibrinogen an ELISA was used [12]. The absorbance was read at 492 nm using a Titertek Multiskan photometer.

For the adhesion assay, purified fibronectin and TBSP diluted in a bicarbonate-coating buffer (15 mM Na_2CO_3, 34.8 mM $NaHCO_3$, pH 9.6) to a concentration of 17 μg/ml, were added separately to 96 microtitre plates with flat-bottom wells (100 μl/well) and incubated 3 h at 37°C. The plates were then washed twice with serum-free medium (MEM) (Gibco). A third wash with serum-free MEM containing 0.5% (w/v) bovine serum albumin was left for 30 min at room temperature. Endothelial cells and smooth muscle cells were harvested from culture by trypsin or collagenase treatment, washed once in serum-free MEM and resuspended at 20000 cells/100 μl per well. The plates were incubated at 37°C. After various incubation times, the non-attached cells were removed and counted with an electronic particle counter. Adherent cells were washed once with serum-free MEM, and trypsinized and counted in a Malassez's cell.

3. RESULTS

Exposure of platelet TBSP (0.2 mg/ml) to thermolysin at a ratio of 1:500 (w/w) for 5 min at 37°C followed by SDS-PAGE under reducing conditions and silver staining resulted in the appearance of major bands at 170, 150, 120, 100, 80, 60, 50, 35 and 30 kDa (fig.1, lane A). After thermolysin treatment of endothelial cell TBSP (0.2 mg/ml), under similar experimental conditions, two silver-stained bands (66 and 18 kDa) present in the endothelial cell TBSP digest were absent from the platelet TBSP digest (fig.1, lane B). On the other hand, two proteolytic bands (100 and 50 kDa) present in the platelet TBSP digest were undetectable in the endothelial cell TBSP digest (fig.1).

After 90 min incubation with thermolysin, silver staining followed by densitography of the electrophoresed endothelial TBSP digest (0.2 mg/ml) showed the disappearance of the undigested TBSP band (170 kDa) with the concomitant appearance of proteolytic bands migrating to 145, 120, 90, 80, 68, 30, 25 and 18 kDa (not shown). Similar proteolytic bands were obtained with the platelet TBSP digest (0.2 mg/ml). However, quantitative differences were demonstrated by densitography (not shown). The endothelial cell TBSP fragment of 80 kDa was decreased by 33% compared to its platelet counterpart whereas the 68 and 18 kDa en-

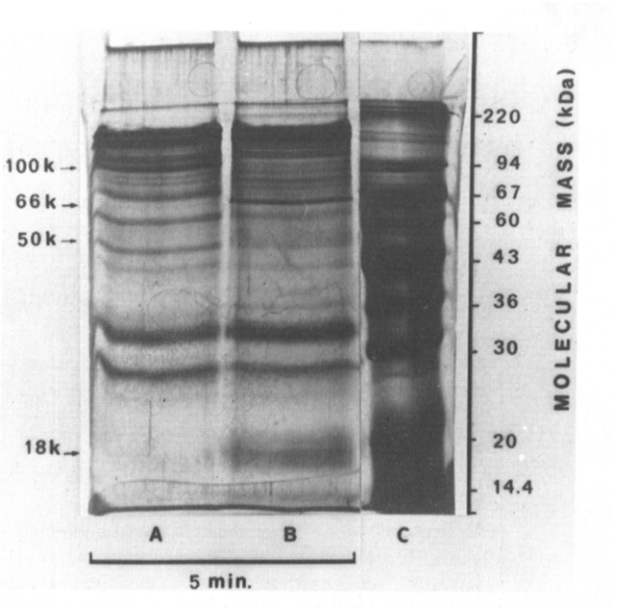

Fig.1. Silver staining of the thermolysin-generated fragments of platelet (A) and endothelial cell (B) TBSP electrophoresed, under reducing conditions, on a 10% SDS-polyacrylamide gel. The same amount of platelet and endothelial TBSP (20 μg) was digested with thermolysin for 5 min at 37°C. (C) High- and low-molecular mass standards.

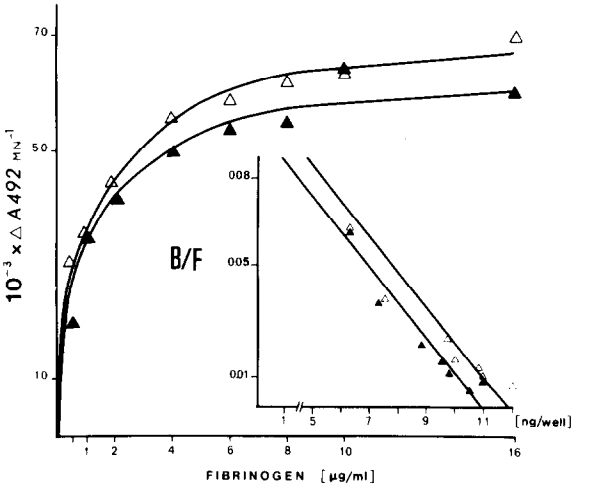

Fig.2. Saturation binding isotherm of fibrinogen to solid-phase adsorbed platelet (▲) and endothelial cell (△) TBSP. The extent of specific fibrinogen complex formation with adsorbed platelet and endothelial cell TBSP was plotted as a function of increasing fibrinogen concentration. Inset: (▲) Scatchard plot of fibrinogen binding to adsorbed platelet TBSP ($y = 0.0124x + 0.134$; $r = -0.970$); (△) Scatchard plot of fibrinogen binding to adsorbed endothelial cell TBSP ($y = 0.013x + 0.153$; $r = -0.934$).

dothelial cell TBSP fragments were increased by 114 and 100%, respectively.

Specific binding of incremental amounts of fibrinogen (0.5–16 μg/ml) to solid-phase adsorbed platelet and endothelial cell TBSP (2 μg/ml) was observed when using an ELISA with an anti-fibrinogen polyclonal antibody (fig.2). The maximum amount of fibrinogen bound to platelet and endothelial cell TBSP was 10.8 and 11.8 ng per well, respectively. The dissociation constants for fibrinogen to platelet and endothelial cell TBSP were 1.86 and 2.4 nM by Scatchard analyses, respectively (fig.2, inset).

Kinetic studies with nine human endothelial cell lines and two human smooth muscle cell lines were performed to assess the adhesion of these cells to

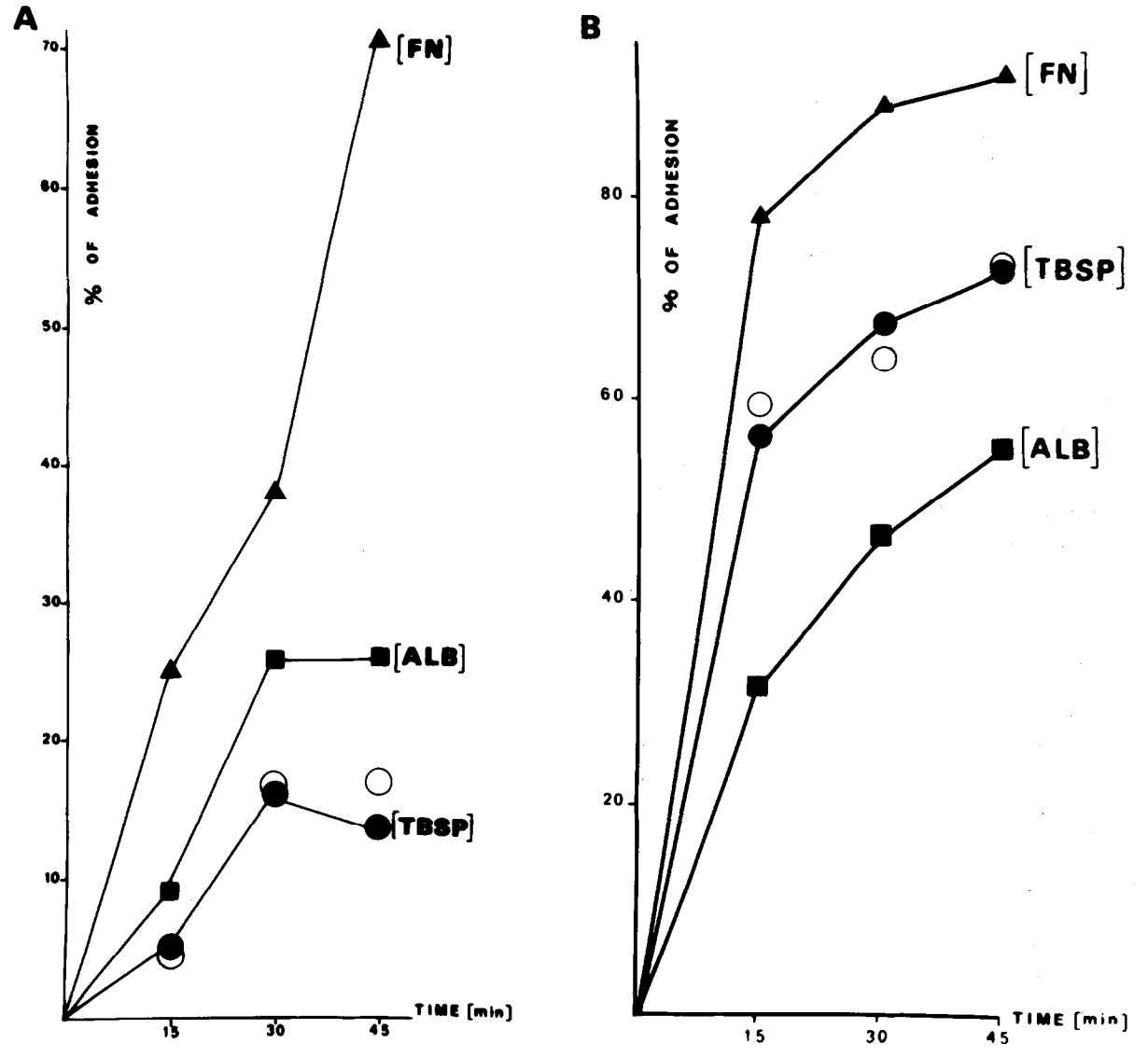

Fig.3. Adhesion assays of human endothelial (A) and smooth muscle cells (B) to solid-phase adsorbed fibronectin (FN), albumin (ALB) and thrombospondin (TBSP). FN (▲), ALB (■), platelet TBSP (●) and endothelial cell TBSP (○), to a concentration of 17 μg/ml, were coated to plastic wells for 3 h at 37°C. Endothelial and smooth muscle cells, harvested from culture by trypsin or collagenase treatment, were incubated (200000 cells/ml) at 37°C. After various incubation times, the non-attached were removed and adherent cells counted.

fibronectin, TBSP and albumin (fig.3). The ability of fibronectin to stimulate adhesion of human endothelial and smooth muscle cells was seen as early as 15 min after plating (fig.3A,B). On the other hand, endothelial cells adhered poorly to endothelial cell and platelet TBSP (fig.3A) whereas smooth muscle cells adhered well (fig.3B).

4. DISCUSSION

Results obtained in this study demonstrate that qualitative and quantitative differences observed between platelet and endothelial cell thrombospondin (TBSP) are centered in two protease-resistant fragments of 68 and 18 kDa. These two fragments may be related to the 70 kDa protease-resistant fragment of TBSP which retains the ability to bind to fibrinogen [6] and to the 18 kDa protease-resistant fragment of TBSP which contains the cell-binding domain [7]. However, the interactions of TBSP with fibrinogen and cultured cells are not affected by the structural differences of platelet and endothelial cell TBSP.

A major feature of platelet and endothelial cell TBSP found in this study is their inability to support endothelial cell adhesion whereas they do for smooth muscle cells. Recently, Lahav et al. [13] and Murphy-Ullrich et al. [14] also reported that platelet TBSP does not promote adhesion of endothelial cells. Since TBSP binds to endothelial cells in suspension [14], it is conceivable that the interaction of TBSP with endothelial and smooth muscle cells occurs via different mechanisms.

Although functional differences regarding the fibrinogen- and cell-binding domains of TBSP were not observed, we have clearly demonstrated that structural differences between platelet and endothelial cell TBSP were present in the 70 and 18 kDa protease-resistant domains of TBSP. These results are similar to those obtained in the study of another multifunctional protein, namely, fibronectin [15]. Functional domains of plasma and cellular fibronectin are also very similar with respect to their affinities for different ligands [15]. However, it is now apparent that plasma and cellular fibronectin are structurally different [16] and it is possible that cellular fibronectin possesses functions that are lacking in plasma fibronectin [17]. Because of the functional similarity between fibronectin and TBSP, it is likely that the structural differences we observed between platelet and endothelial cell TBSP may lead to functional differences. However, the TBSP functions originating from these structural differences are not yet known. Further work is in progress to clarify the structure-function relationship between platelet and endothelial cell TBSP.

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